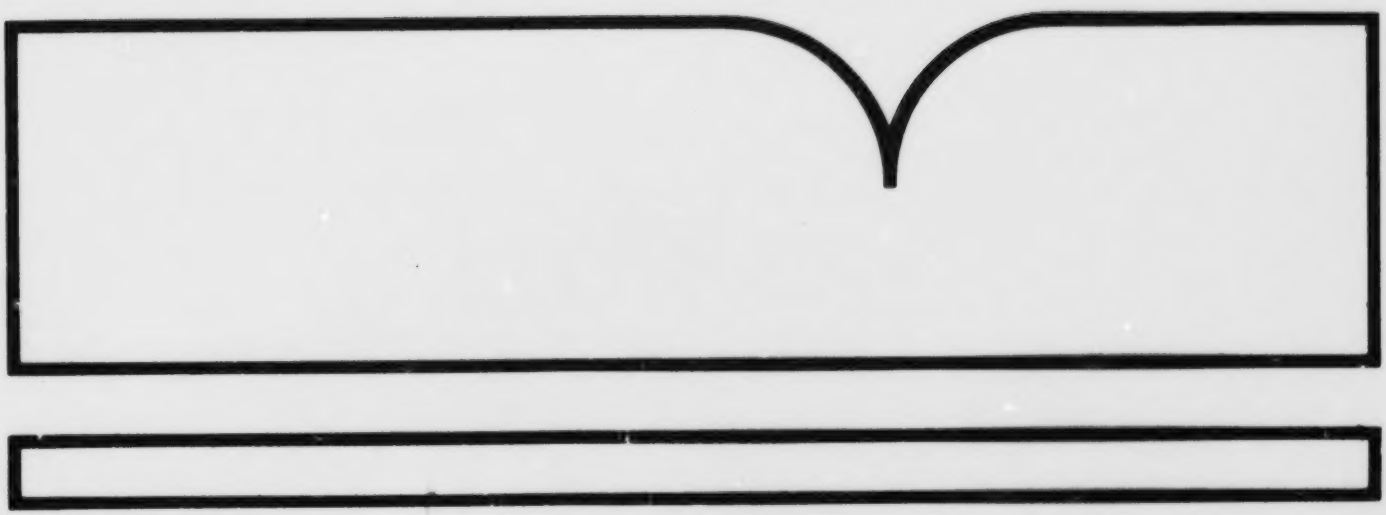
PB84-144336

Effect of Incubation and Activation Conditions on the Hepatocyte-Mediated Plate Incorporation and Preincubation 'Salmonella typhimurium' Mutagenesis Assays

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1983



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TECHNICAL REPORT DATA
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1. REPORT NO.	2.	3. RECI PB84-144336
EPA-600/J-83-137		

4. TITLE AND SUBTITLE
Effect of Incubation and Activation Conditions on the Hepatocyte-Mediated Plate Incorporation and Preincubation Salmonella typhimurium Mutagenesis Assays(Journal Version)

5. REPORT DATE
1983

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
GeneticiBioassay Branch/Genetic Toxicology Division
Health Effects Research Laboratory
US Environmental Protection Agency
Research Triangle Park, NC 27711

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Office of Research & Development
Health Effects Research Laboratory
US Environmental Protection Agency
Research Triangle Park, NC 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA-600/11

15. SUPPLEMENTARY NOTES
REF: Teratogenesis, Carcinogenesis, and Mutagenesis, 3:367-376, 1983

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	11
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

EPA-600/J-83-137
JOURNAL ARTICLE

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Effect of Incubation and Activation Conditions on the Hepatocyte-Mediated Plate Incorporation and Preincubation *Salmonella typhimurium* Mutagenesis Assays

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Primary cell-mediated microbial mutagenesis assays have been shown to be useful in detecting specific target organ genotoxic activity. The lack of a standard protocol for these assays, however, makes interlaboratory comparisons difficult. In order to standardize the hepatocyte-mediated *Salmonella typhimurium* mutagenesis assay, incubation and activation conditions for the plate incorporation and preincubation assays were examined using two aromatic amines, 2-aminofluorene (AF) and 2-acetylaminofluorene (AAF). Direct comparison of two preincubation protocols demonstrated the necessity for the hepatocytes to be present during the two- to three-day plate incubation period. An examination of various preincubation times showed relatively minor differences between 15 and 90 minutes. The preincubation and plate incorporation protocols were directly compared using both hamster and rat hepatocytes. For both preincubation and plate incorporation, the optimum concentration of hepatocytes was shown to be 1×10^6/plate. Direct evaluation of various hepatocyte-mediated bacterial protocols should facilitate future interlaboratory comparisons using a more standardized procedure.

Key words: hepatocytes, microbial mutagenesis, *Salmonella typhimurium*, 2-aminofluorene, 2-acetylaminofluorene, hepatocyte-mediated mutagenesis

INTRODUCTION

Whole cells such as hepatocytes are now generally recognized as providing a metabolism system that is more similar to cellular metabolism in intact animals than liver S9 preparations. Carcinogen metabolite studies [1–3] as well as DNA adduct studies [4,5] have demonstrated the advantages of using cellular metabolism systems. Whole liver cells contain not only the oxidative enzyme systems needed to activate

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some promutagens [6,7], but also the acetylation [8] and conjugation [9] enzymes normally present in intact tissue.

Several studies have demonstrated the utility of hepatocytes as a metabolic activation system for *Salmonella typhimurium* mutagenicity assays [10–12]. In these separate studies, both preincubation [1,13] and plate incorporation [11,12] techniques were employed; however, these assay conditions were not directly compared. Dybing et al [1] and Staiano et al [13] both reported the use of a preincubation technique in which the hepatocytes are removed prior to plating. However, Staiano et al [13] reported substantially less (7×) mutagenic response with this technique. Our examination of these various techniques showed significant differences as a result of varying the preincubation and assay conditions.

In order to standardize the protocol for the hepatocyte-mediated plate incorporation and preincubation assays, we have examined various incubation and activation conditions with both hamster and rat hepatocytes. This paper reports the comparison of these parameters and assays using two aromatic amines, 2-aminofluorene[1] (AF) and 2-acetylaminofluorene[2] (AAF). These compounds were selected due to comparative data available in the literature and the significant differences observed between rat and hamster hepatocyte activation [1,11].

MATERIALS AND METHODS

Chemicals

The aromatic amines 2-aminofluorene and 2-acetylaminofluorene were obtained from Aldrich Chemical Company (Milwaukee, WI). Aroclor 1254 was purchased from Monsanto (St. Louis, MO). Spectrograde dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Company (Raleigh, NC).

Animals

Adult male Sprague-Dawley rats (250–300gm) from Charles River Breeding Laboratory (Wilmington, MA) and adult male Syrian golden hamsters (90–150gm) from Engle Laboratory (Farmersburg, ID) were used for the preparation of hepatocytes. The animals were maintained on Purina lab chow (Ralston Purina Company, St. Louis, MO) and water ad libitum. The animals were housed in an animal room with a 12 hr light/12 hr dark cycle, and hepatocytes were isolated within 2–3 hours after initiation of the light cycle. For induction of animals, a single dose of Aroclor 1254 (500mg/kg body weight) in corn oil was injected intraperitoneally five days before sacrifice. Food was withheld for the 24 hours before isolation of hepatocytes.

Preparation of Isolated Hepatocytes

The perfusion method described by Williams et al [14] and modified by Inmon et al [15] was used for the isolation of both rat and hamster hepatocytes. Cell viability was 78% to 92% as determined by trypan blue exclusion. Hepatocytes were suspended in sterile PBS (Dulbecco's Ca- and Mg-free phosphate-buffered saline; Grand Island Biological Company, Grand Island, NY) at a concentration of 5×10^6 hepatocytes/ml.

The amount of histidine present in uninduced rat hepatocytes was assayed by determining fluorescamine derivatives [16].

[1]CAS #153786.

[2]CAS #53963.

Preparation of Bacteria

Salmonella typhimurium strain TA98 was obtained from Dr. Bruce N. Ames (University of California, Berkeley). A single colony isolate was used to inoculate an overnight broth culture. This culture was centrifuged at 2,000 rpm for 20 minutes in a refrigerated Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Newton, CT). The bacterial pellet was resuspended in sterile PBS and diluted to a concentration of 15×10^8 bacteria/ml.

Preincubation Assay

Preincubation conditions that were examined in this study included centrifugation to remove the hepatocytes before plating for revertant colony growth, length of preincubation time, the effect of Aroclor induction on the hepatocytes, and the concentration of hepatocytes.

Preincubation mixtures consisted of the following: 1.0 ml Ames buffer (17), 0.1 ml TA98 (1.5×10^8 bacteria), 0.8 ml hepatocytes in PBS (generally, 4×10^6 cells), and AAF or AF in 0.1 ml DMSO to give final concentrations of 0.05, 0.1, 0.25 or 0.5 mM in a total volume of 2 ml.

These mixtures were prepared in 15-ml sterile, conical-bottom centrifuge tubes (Corning, Fisher Scientific Company, Raleigh, NC) on ice and then transferred to a controlled-environment incubator/shaker (New Brunswick Scientific Company, New Brunswick, NJ) set at 37°C and 100 rpm. At the end of preincubation, the tubes were returned to ice. An aliquot (0.5 ml) was added to 2 ml of molten top agar and poured onto Vogel-Bonner minimal media (VBM) plates [17]. Triplicate plates were prepared from each tube. In order to determine the number of bacterial survivors, 0.5 ml of a 10^6 dilution prepared from each tube was added to 2 ml of molten top agar containing 78 μg of histidine. This mixture was poured onto VBM plates. Triplicate plates were prepared from each dilution tube. All plates were incubated at 37°C for 72 hours in a Precision mechanical convection incubator (Precision Scientific Group, Chicago, IL). Revertant colonies for each plate were counted on an automatic colony counter (Artek System Corporation, Farmingdale, NY).

Centrifugation, when used, was carried out in an IEC HN-S tabletop centrifuge (Damon/IEC Division, Needham Heights, MA) according to the method of Dybing et al [1]. In order to sediment the hepatocytes, the tubes were centrifuged at 600 rpm ($55 \times g$) for one minute; the supernatant was pipetted into another sterile tube and spun at 3,000 rpm ($880 \times g$) for 15 minutes to pellet the *Salmonella*. The bacteria were resuspended in 2 ml of sterile PBS by gentle agitation and plates were poured using 0.5-ml aliquots as described above.

Preincubation times of 15, 30, 60, and 90 minutes were compared. When the effect of hepatocyte concentration was examined, tubes were prepared with 0.8, 0.4, and 0.08 ml of a stock concentration of 5×10^6 hepatocytes/ml PBS to give concentrations, respectively, of 4×10^6, 2×10^6, and 4×10^5/tube (1×10^6, 5×10^5, and 1×10^5/plate).

Plate Incorporation Assay

For plate incorporation assays, tubes containing mixtures of hepatocytes, *Salmonella*, and AAF or AF were prepared as described for the preincubation assays.

The tubes remained on ice until plates were poured from 0.5-ml aliquots. This method of plate incorporation assay was compared with the standard Ames plate incorporation assay method [17] of adding bacteria, test substance, and metabolic activator (in this case, the hepatocytes) individually to the molten top agar; no significant difference was found. The method described above allowed duplicate tubes to be prepared for preincubation and plate incorporation in the same experiment using the same hepatocyte, bacteria, and test compound stock solutions for both assays.

RESULTS

Various hepatocyte-mediated assay parameters were compared in order to standardize the assay procedure for both the plate incorporation and the preincubation techniques. The effect of centrifuging to remove the hepatocytes after the 30-minute preincubation period is shown (Fig.1) for AAF and AF using induced hamster hepatocytes. For both AAF and AF, the number of revertants/plate was greatly diminished following centrifugation. The increased activity observed when the hepatocytes were not removed by centrifugation was not due to increased amounts of histidine present on the plates (6.2 μg/1 $\times$ 10^6 hepatocytes), as this concentration did not increase the spontaneous rate of revertant colony formation in control incubations. Representative colonies from each treatment were shown to be true revertants by growth on histidine-free biotin plates. All other experiments were therefore performed without removal of the hepatocytes.

Another condition of preincubation examined was the length of preincubation time. There was no appreciable difference in response for up to 60-minute preincubation of uninduced hamster hepatocytes with either AAF or AF (Fig. 2). However, after 90 minutes, there appeared to be a slight diminution in activity, especially with AF, and a decrease in survivors. Therefore, 30 minutes was selected as the standard preincubation time.

Comparison of Aroclor-induced hamster hepatocytes with uninduced hamster hepatocytes (Fig.3) shows that induced hepatocytes were significantly more active than uninduced hepatocytes with AAF, but there was no significant difference between the two with AF. With rat hepatocytes, increased activity with induction was observed with AF but not with AAF (data not shown). The induced rat hepatocytes, however,

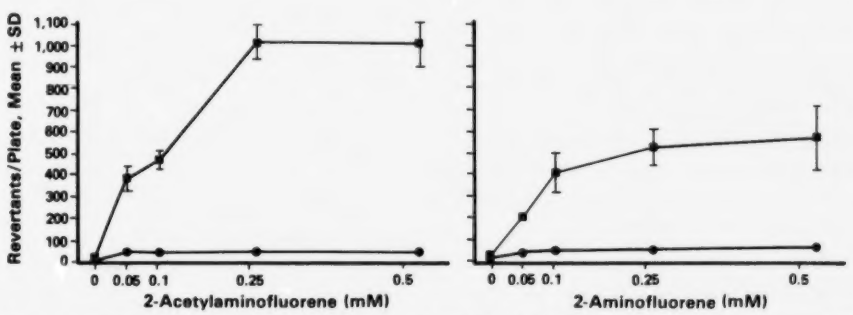

Fig. 1. Comparison of centrifuging (●) and not centrifuging (■) in the preincubation assay with Aroclor-induced hamster hepatocytes.

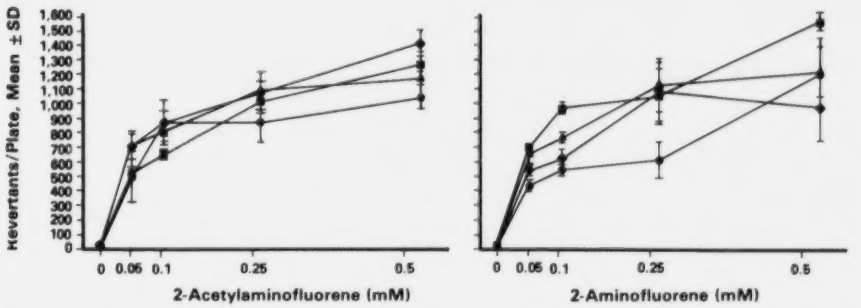

Fig. 2. Varying preincubation times with uninduced hamster hepatocytes. (■) 15 minutes, (▲) 30 minutes, (◆) 60 minutes, and (●) 90 minutes.

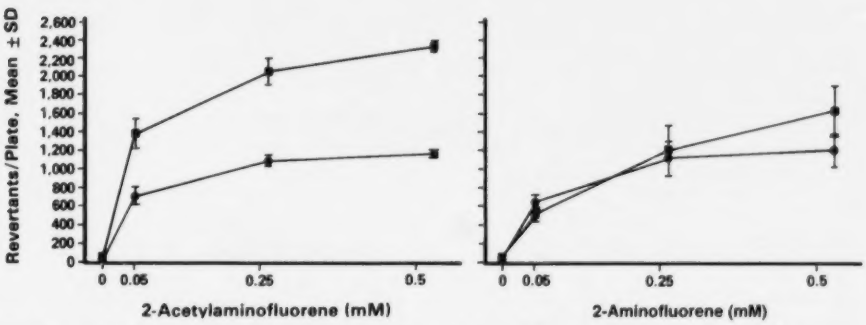

Fig. 3. Comparison of Aroclor-induced (■) and uninduced (●) hamster hepatocytes in the preincubation assay.

showed at least an order of magnitude less activity than the hamster hepatocytes for both compounds (Table I).

The effect of hepatocyte concentration was examined for both preincubation and plate incorporation using induced hamster hepatocytes (Fig. 4). The concentration of 1×10^6 hepatocytes/plate resulted in the most activity for both compounds. Comparison with a plate incorporation assay showed no significant differences with either AAF or AF, 1×10^6 hepatocytes/plate yielding the highest number of revertants. The addition of higher concentrations of hepatocytes (up to 5×10^6/plate) did not result in consistently higher activity due to nonuniform suspension of the hepatocytes.

The preincubation and plate incorporation techniques were also compared using both induced hamster and induced rat hepatocytes (Table I). The use of hamster hepatocytes resulted in significantly higher mutagenic activity for both AAF and AF, compared to the rat hepatocytes. With hamster hepatocytes, the number of revertants was slightly higher with the plate incorporation assay than with the preincubation assay. More activity was seen with AAF than AF when hamster hepatocytes were the source of activation; the reverse occurred when rat hepatocytes were used.

TABLE I. Preincubation and Plate Incorporation Assays with Hamster or Rat Hepatocytes

		Rev/plate[a]			
		Preincubation		Plate incorporation	
	mM	Hamster[b]	Rat[b]	Hamster[b]	Rat[b]
2AAF	0	30.7 (2.1)	100.7 (12.1)	24.7 (5.9)	71.7 (10.7)
	0.05	889.7 (113.4)	103.5 (6.4)	1,224.3 (103.9)	103.7 (26.7)
	0.10	1,480.0 (74.1)	64.0 (6.9)	1,904.3 (77.9)	112.7 (37.1)
	0.25	1,987.0 (83.1)	93.3 (32.8)	2,335.0 (64.8)	96.3 (17.5)
	0.50	1,952.0 (9.9)	74.0 (2.7)	2,410.3 (107.6)	75.0 (5.7)
Slope[c]		14917.9	ND[d]	11605.4	333.9
2AF	0	30.7 (2.1)	100.7 (12.1)	24.7 (5.9)	71.7 (10.7)
	0.05	578.0 (84.1)	77.7 (15.4)	754.7 (54.0)	75.0 (0)
	0.10	976.7 (122.9)	90.0 (18.2)	1,249.3 (126.1)	80.3 (18.6)
	0.25	1,213.0 (43.2)	119.3 (27.4)	1,814.7 (218.8)	145.3 (48.7)
	0.50	1,645.7 (137.2)	146.3 (28.1)	1,935.0 (30.4)	190.0 (66.4)
Slope[c]		4209.9	1109.2	12180.9	1249.1

[a]Mean (± SD) of triplicate plates.
[b]Animals were induced with Aroclor as described in Materials and Methods.
[c]Rev/mM: Nonlinear model slope by the method of Stead et al [20].
[d]ND: Not determinable.

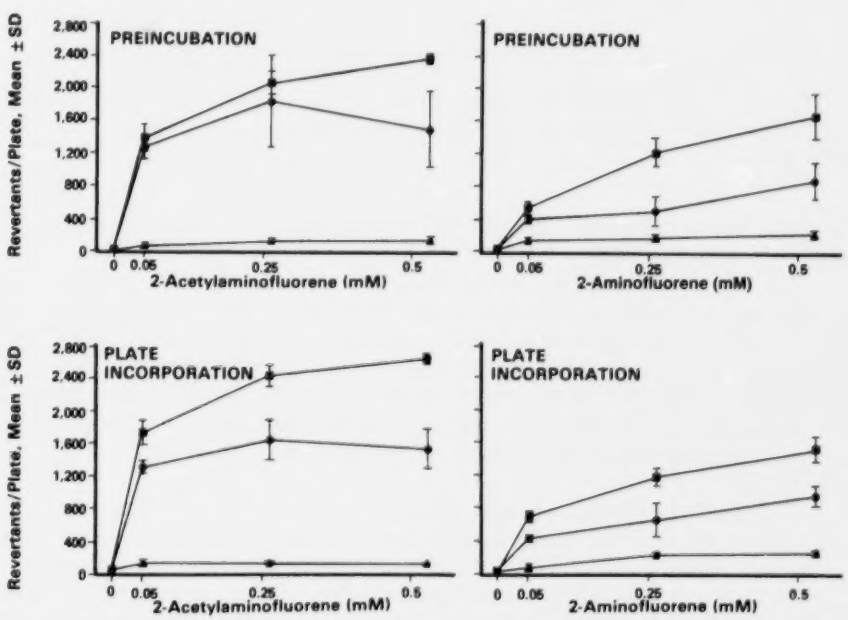

Fig. 4. The effect of hepatocyte concentration in the preincubation and plate incorporation assays. (▲) 1×10^5, (●) 5×10^5, (■) 1×10^6 hepatocytes/plate.

Finally, the ability of Aroclor-induced hamster and rat hepatocytes to act as metabolic activators of AAF and AF in the Ames plate incorporation assay were compared to that of the classic, Aroclor-induced rat liver S9 system (Table II). With AF, the standard S9 provided optimum mutagenic activity; with AAF, the hamster hepatocytes resulted in the highest mutagenic activity observed. Rat hepatocytes generally showed much less (an order of magnitude) activity than hamster hepatocytes. In these experiments, the S9 and hepatocyte concentrations were selected to provide optimum activity with AF and AAF rather than equivalent protein concentrations because the purpose was to compare their respective abilities as metabolic activators under optimum conditions.

DISCUSSION

Evaluation of the incubation and activation conditions for both hepatocyte-mediated plate incorporation and preincubation assays demonstrates that both of these assays are useful and can, with certain compounds, yield similar results. In the preincubation assay, however, these studies show that the hepatocytes must also be incorporated into the top agar for optimal activity. Although Dybing et al [1] reported high mutagenic activity for AAF after removing the hepatocytes before plating, a subsequent paper by Staiano et al [13], in which the hepatocytes were removed, following Dybing's method, reported lower activities that are similar to those described here. The decreased activity could also be due to removal of active metabolites from the media as a result of centrifugation to pellet the bacteria. However, several independent investigations using plate incorporation assays with hepatocytes incorporated into the top agar also support the need for hepatocytes to be present during the two to three days of incubation for optimal activity [11,12].

TABLE II. Comparison of Hamster Hepatocytes, Rat Hepatocytes, and Rat Liver S9 in the Plate Incorporation Assay

		Rev/plate[a]		
		Hepatocytes		S9
	mM	Hamster[b]	Rat[b]	Rat[b]
2AAF	0	42.5 (19.0)	31.0 (5.3)	42.3 (7.1)
	0.1	1,733.3 (143.2)	29.3 (6.7)	848.0 (68.5)
	0.2	2,429.3 (133.1)	—	1,561.7 (48.0)
	0.5	2,642.3 (56.9)	34.3 (5.5)	1,613.0 (97.6)
	0.9	—	35.7 (13.7)	2,114.0 (157.0)
Slope[c]		5,394.7	ND[d]	3,463.8
2AF	0	31.3 (12.1)	39.0 (2.7)	42.3 (7.1)
	0.1	687.3 (58.2)	—	2,311.3 (196.8)
	0.2	1,170.0 (104.9)	98.7 (15.1)	2,894.7 (121.8)
	0.5	1,506.0 (153.4)	138.0 (9.9)	2,781.3 (177.9)
	1.0	—	187.7 (8.7)	2,188.7 (51.6)
Slope[c]		3,065.5	148.2	9,884.6

[a]Mean (>SD) of triplicate plates.
[b]Animals were induced with Aroclor as described in Materials and Methods.
[c]Rev/mM: Nonlinear model slope by the method of Stead et al [22].
[d]ND: Not determinable.

The optimum preincubation time selected in the studies reported here is 30 minutes. Although very little difference was observed between 15 and 60 minutes for both compounds over the entire concentration range tested, 90 minutes tended to result in lower mutagenicity. This is consistent with previous observations that the metabolic capabilities of hepatocytes are significantly decreased the longer they are incubated in suspension [2].

Poiley et al [11], using high doses of AAF above the linear portion of the dose-response curve, reported that the Aroclor 1254 induction of both rats and hamsters resulted in hepatocytes that gave higher numbers of revertants than hepatocytes from uninduced animals. At these high doses, we also observed that AAF gives higher revertants/plate when the hepatocytes are obtained from Aroclor-induced hamsters. Examination of the data from the entire concentration range, however, shows no significant increase in mutagenic activity as a function of hamster induction at the lower doses of AF and AAF, which are in the linear portion of the dose-response curve. Since hamster hepatocytes as well as hamster S9 are generally more metabolically active than rat hepatocytes or rat S9, with or without Aroclor 1254 induction [18], a direct comparison of rat and hamster hepatocytes is reported here only with induction. In all cases with these compounds, higher mutagenic activities resulted with the hamster hepatocytes. This observation is consistent with the studies of Raineri et al [18] comparing induced and uninduced hamster and rat S9.

With both the preincubation and plate incorporation assays, 1×10^6 hepatocytes/plate resulted in the highest mutagenic activity for both compounds. Higher concentrations of hepatocytes (5×10^6) did not consistently result in higher activity. Poiley et al [11], using only the plate incorporation assay, also generally found that 1×10^6 hepatocytes from both induced and uninduced male hamsters gave the highest number of revertants.

Comparison of the preincubation and plate incorporation protocols showed very little difference between these two assays with AF and AAF. Because some compounds such as dimethylnitrosamine (DMN) show more activity in the preincubation assay [18,19], nitrosamines and complex mixtures should be tested with the preincubation protocol as well as the plate incorporation assay.

We have not yet investigated the quantitative differences between hepatocyte and S9 activation for both rats and hamsters in the studies reported here. It is apparent, however, from comparing our data with that of Raineri et al [18] that hepatocytes from either rats or hamsters yield less mutagenic activity with 2AAF than S9 preparations from the corresponding species. One of the important features of whole cell activation is the presence of intact cellular membranes and metabolism systems that include both the activation and detoxification enzymes present in intact tissue in the animal. In the case of 2AAF, Stout et al [21] have shown that the principal mutagenic metabolite formed by liver S9 is N(OH)2AF. This metabolite is formed by microsomal oxidation of 2AAF to N(OH)2AAF, followed by deacetylation by the cytosol fraction to N(OH)AF. Brouns et al [6] have shown that the same deacetylation reaction occurs in intact hepatocytes to produce the mutagen N(OH)AF; however, whole cells also esterify the hydroxamic acid and form a sulfate ester that appears to result in DNA damage inside the liver cell but does not contribute to the mutagenic activity observed when hepatocytes are used for the activation system with *S typhimurium*. Since the sulfate ester appears to remain inside the hepatocyte, this compet-

ing sulfation may account for the lower bacterial mutagenicity observed with hepatocyte compared to S9 activation.

ACKNOWLEDGMENTS

The authors would like to thank Robert Langenbach of the U.S. Environmental Protection Agency for helpful discussions and suggestions during the course of this work. The authors would also like to thank Joan Croteau of Northrop Services, Inc., for editing this manuscript.

Information in this article has been cleared by the Health Effects Research Laboratory and approved for publication.

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